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Exercise Training and Losartan Improve Endothelial Function in Heart Failure Rats by Different Mechanisms

Short title: Exercise and losartan in heart failure

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ABSTRACT

Objectives: To investigate the mechanisms of losartan- and exercise training-induced improvements on endothelial dysfunction in heart failure.

Design: Sprague-Dawley rats subjected to left coronary artery ligation inducing myocardial infarction and heart failure were randomized to losartan treatment, high-intensity exercise training, or both.

Results: Losartan, but not exercise training, reduced the heart failure-associated elevation in left ventricular end-diastolic pressure (26 ± 2 mmHg vs. 19 ± 1 mmHg after losartan). In contrast, both exercise training and losartan improved exercise capacity, by 40% and 20%, respectively; no additional effects were observed when exercise training and losartan were combined. Aortic segments were mounted on a force transducer to determine vasorelaxation. Heart failure impaired endothelium-dependent vasorelaxation, observed as a 1.9-fold reduced response to acetylcholine (EC_{50}). Exercise and losartan improved acetylcholine-mediated vasorelaxation to the same extent, but by different mechanisms. Exercise training upregulated the nitric oxide pathway, whereas losartan upregulated a non-nitric oxide or -prostacyclin pathway; possibly involving the endothelium-dependent hyperpolarizing factor.

Conclusions: Both losartan and exercise training reversed endothelial dysfunction in heart failure; exercise training via nitric oxide-dependent vasorelaxation, and losartan via an unknown mechanism that may involve endothelium-dependent hyperpolarizing factor. Thus, the combined treatment activated an additional nitric oxide-independent mechanism that contributed to reduce endothelial dysfunction.

INTRODUCTION

Endothelial dysfunction contributes to heart failure (HF) pathogenesis by increasing cardiac afterload and reducing muscle perfusion with subsequently reduced exercise capacity (1,2). It is characterized by a reduced capacity of the blood vessels to dilate and is caused by reduced availability of substances causing vascular smooth muscle relaxation. The main smooth muscle relaxant is endothelium-derived nitric oxide (NO), of which generation may be limited by substrate for production (L-arginine), limitation and deactivation of the catalyzer (endothelial NO synthase, eNOS) and scavenging of NO (oxidant status). However, independent of NO, also insufficient availability of endothelial-derived hyperpolarizing factors (EDHF) can contribute to decreased smooth muscle relaxation (7), although the exact cellular mechanism by which this occurs remains unknown.

As such, restoration of endothelial function remains important for the management of HF. Both physical exercise and the angiotensin II type 1 receptor blocker (ARB) losartan are known to beneficially alter endothelial and overall health (4,5). Exercise training counteracts endothelial dysfunction by activating NO production by eNOS (1), and by improving work capacity and quality of life in HF patients (2,5-7), whereas losartan also favorably affects the outcome of HF (8,9), at least partly by enhancing endothelial-dependent vasorelaxation (10). Thus, both exercise training and losartan treatments may be advantageous for vascular and endothelial health in HF. However, the effect of a combination of exercise training and losartan in HF relative to treatments with either option alone remains poorly understood.

The aim of this study was therefore to determine the effect of exercise training and losartan, either each alone or in combination, on the endothelial dysfunction in HF after myocardial infarction. Moreover, we also investigated the mechanisms by which endothelial dysfunction

was corrected. Our hypothesis was that both exercise training and losartan would contribute toward correcting endothelial function in HF, but by different endothelium-dependent mechanisms.

MATERIALS AND METHODS

Study design

Left coronary artery ligation leading to myocardial infarction and subsequent HF, or sham operation, as previously described (11,12), was performed in female Sprague-Dawley rats (Møllegaards Breeding Center Ltd, Denmark) during 1% isoflurane anaesthesia in 70% O₂/30% N₂O. Buprenorphine (0.05mg Temgesic, Reckitt and Coleman, Hull, UK) was given subcutaneously immediately and 10 hours after surgery. 7 days post-surgery, 2-dimensional short-axis echocardiography confirmed myocardial infarction. Rats were randomized to 6 different groups with 8 in each group; see Table 1A. Losartan (2 g·L⁻¹ *ad libitum*, Merck & Co., Whitehouse Station, NJ) or placebo in the drinking water was initiated 1 week after the surgery, whereas exercise training was initiated 4 weeks after the surgery, to mimic a clinically relevant treatment strategy; controls remained sedentary. 48 hours after the last exercise session, subcutaneous anaesthesia was given (in mL: 0.33 haloperidol, 0.5 fentanyl, 0.5 midazolam, 0.3 ketamine hydrochloride, 0.5 water) and a pressure microtip catheter (2-Fr size Millar, Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced to the heart, whereby left ventricular (LV) end-diastolic and peak systolic pressures were measured, before euthanasia. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and was approved by the Institutional Review Board.

Maximal oxygen uptake and exercise training

After a 20-minute warm-up by running on a 25° inclined treadmill at ~50% of the maximal oxygen uptake (VO_{2max}), the treadmill velocity was increased progressively every 2 minutes until exhaustion. Oxygen uptake was measured continuously in a custom-made metabolic chamber, and the highest recorded value was taken as a representation of VO_{2max}.

The endurance exercise training was performed as previously described (11,12). After 10-minutes of warm-up, rats were running uphill (25°) on a treadmill for 1.5 hours, alternating between 8 minutes at an exercise intensity corresponding to 85-90% of $\text{VO}_{2\text{max}}$, and 2 minutes active recovery at 50-60%. Exercise was performed 5 days/week for 8 weeks, and $\text{VO}_{2\text{max}}$ was measured every week in the exercising animals to adjust running speed in order to maintain high intensity throughout the exercise period, whereas $\text{VO}_{2\text{max}}$ in sedentary rats was measured before and after the experimental period.

Vascular function

The abdominal aorta was carefully dissected with care taken to not touch the endothelium. 6 2-3 mm long segments from each rat were connected to force transducers and immersed in a 10 mL organ bath containing Krebs buffer, whereupon tension was increased stepwise to 1 gram over 75 minutes. A modified high K^+ (60 mM) Krebs buffer was added and washed out again, and tension was again stabilized to 1 gram, whereby a dose of phenylephrine ($3 \cdot 10^{-4}$ M) was added before addition of acetylcholine (10^{-4} M) to ensure reactivity. After washing and stabilizing the segments once more, the following protocols A-F were initiated; see Table 1B. Briefly described, aorta segments were contracted with phenylephrine and relaxed with accumulating doses of either acetylcholine or Na^+ nitroprusside (SNP), in the presence of superoxide dismutase (SOD) and/or indomethacin to inhibit superoxide and prostacyclin productions, respectively. After re-stabilizing and incubating the segments as described above, a second set of contractions and relaxations were initiated, but this time with the addition of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) or N^{G} -nitro-L-arginine (LNNA) for 45 minutes to inhibit soluble guanylyl cyclase (sGC) and eNOS, respectively (Table 1B). The chemicals were purchased from Sigma-Aldrich, except ODQ (Cayman Chemical, Ann Arbor, MI). Dose-response curves and EC_{50} -values (the agonist concentration

that evokes 50% of maximal dilatation) were obtained and areas under curves were calculated using the trapezoidal rule in Sigmaplot version 8.02. Although the abdominal aorta is a capacitor, and has little importance in the regulation of blood pressure, previous studies in our group have shown equal exercise-induced endothelial responses in the carotid artery as in the aorta (13).

Statistical analysis

Mann-Whitney U test determined differences in EC_{50} -values, and a univariate repeated-measures general linear model with the Scheffé post hoc test assessed differences in the relaxation responses. To identify changes in VO_{2max} , the Friedman test for within-group comparisons over time and the Kruskal-Wallis test for between-group comparisons were used. $p < 0.05$ was considered as significant. Data are presented as mean \pm SEM.

RESULTS

HF

In order to confirm development of HF after myocardial infarction due to left coronary artery ligation, we measured LV end-diastolic and peak-systolic pressures (LVEDP and LVPSP, respectively). We observed elevated LVEDP and reduced LVPSP in all post-myocardial infarction rats included in the study, and thus the presence of HF was confirmed (Table 2). Exercise training did not alter the LVEDP, although a small but insignificant reduction was noted. In contrast, losartan decreased LVEDP by 8 mmHg. None of the treatments corrected for abnormal LVPSP.

VO_{2max}

HF was associated with a ~40% reduction in exercise capacity (VO_{2max}) as compared to healthy sham-operated rats (Fig. 1). However, exercise training restored VO_{2max} to levels comparable to sedentary sham-operated rats. The magnitude of the exercise effect was similar to that of healthy rats. Losartan alone also improved VO_{2max}, but considerably less than exercise training (~20%), whereas the combination of losartan and exercise training did not result in any effect beyond that of exercise training alone. This suggests that exercise training may take out the potential for improvement, such that additive strategies for increasing VO_{2max} have no further effects. Both sham-operated and HF rats that remained sedentary and did not receive losartan decreased VO_{2max} over the course of the study.

Acetylcholine-dependent vasorelaxation

Endothelium-dependent vasorelaxation in the aorta was reduced in HF; indicating endothelial dysfunction, as evidenced by the 1.9-fold higher EC₅₀, i.e. reduced sensitivity, in the response to acetylcholine in HF compared to sham-operated rats (Fig. 2A and B). Exercise training,

losartan, and the combination of losartan and exercise training improved vasorelaxation 2.8-fold, 3.3-fold, and 3.4-fold, respectively ($p < 0.05$ for all); thus, the combination of exercise training and losartan did not change the magnitude of improvement compared to either intervention alone. Furthermore, aorta segments of HF rats on losartan or exercise training also showed a trend toward higher sensitivity to acetylcholine than those of sedentary healthy rats, albeit it did not reach statistical significance. Exercise training in healthy rats also improved the acetylcholine sensitivity (EC_{50}) and hence endothelial function. Maximal dilatation (V_{max}) of the aorta tended to be reduced in HF (~18%, $p < 0.1$), but restored by treatment with both losartan and exercise training (Fig. 2C).

Acetylcholine-mediated vasorelaxation after addition of L-arginine and SOD

Addition of 3 mM L-arginine improved vasorelaxation in untreated, sedentary HF rats, observed as a 1.9-fold increase in acetylcholine sensitivity (Fig. 2D). L-arginine-dependent effects were not seen in the other groups, although EC_{50} tended to improve in sham-operated sedentary rats, but this did not reach statistical significance ($p = 0.06$).

Incubation with 250 IU SOD increased sensitivity to acetylcholine in untreated, sedentary HF and sham-operated rats by 3.4-fold and 2.3-fold, respectively (Fig. 2E). No significant changes occurred in the remaining groups, although a trend for improved endothelial function was observed in all of them.

Addition of L-arginine or SOD did not affect V_{\max} in any of the groups (data not shown). Nonetheless, as shown in Fig. 2D and E, HF presents with a potential for improving acetylcholine-mediated vasorelaxation by interventions to increase L-arginine or to inhibit superoxides, which react with NO to form peroxynitrite. This potential is no longer observed in HF after treatment with exercise training or losartan.

Acetylcholine-mediated vasorelaxation after inhibition of prostacyclin and NO productions

Incubation with 10^{-5} M indomethacin revealed no further differences in vasorelaxation between the groups (Fig. 3A), suggesting that prostacyclin did not contribute to the observed vasorelaxation effects presented in Fig. 2. In contrast, inhibition of NO-synthesis by 10^{-4} M LNNA abolished the exercise-induced increase in vasorelaxation, whereas losartan-treated rats remained with a trend ($p=0.12$) towards greater vasorelaxation (Fig. 3B). Moreover, blocking sGC with 10^{-5} M ODQ before adding accumulating doses of acetylcholine abolished vasorelaxation and instead lead to a minor, but uniform contraction of 2-4% (Fig. 3C).

Subtracting the area under these two curves from the area under the curve of the non-NO-inhibited relaxation (curve 3A-(curves 3B+3C)) revealed that the exercise trained aorta segments relied ~50% more on NO for relaxation, compared to the sedentary groups (Fig. 3D). This was observed in both sham-operated and HF rats. These pharmacological

maneuvers also suggest that losartan in contrast exerted its effect on endothelium-dependent vasorelaxation through a non-NO and non-prostacyclin-mediated effect; of which molecular identity remains unknown, but may involve EDHF. The reason this suggests EDHF as the mechanism is that it is the only known vasorelaxant downstream of acetylcholine and independent of NO and prostacyclin. As such, treatment with either losartan or exercise training reversed the endothelial dysfunction, but by different mechanisms, which were not additive when combined, suggesting a ceiling effect on endothelial-dependent vasorelaxation of either treatment alone.

Finally, incubation with 250 IU SOD significantly ($p < 0.05$) increased the LNNA/indomethacin-resistant vasorelaxation in all groups except sedentary HF rats on losartan (data not shown), whereas adding 10^{-4} M SNP to aorta segments incubated with LNNA and ODQ lead to ~100% and ~0% relaxation, respectively (data not shown).

DISCUSSION

HF was associated with endothelial dysfunction in the abdominal aorta. However, the dysfunction was largely reversed by exercise training or by treatment with the ARB losartan, but by two distinct signaling pathways; exercise training through an NO-dependent effect and losartan through a non-NO- and non-prostacyclin effect; thus suggesting losartan may have activated EDHF. When both exercise training and losartan were combined, losartan did not inhibit the salutary effects of NO, and furthermore, it activated an additional pathway of vasoregulation that may become important in periods of exercise cessation, albeit the combination did not improve endothelial-dependent vasorelaxation further of either treatment alone. This may be explained by exercise training and losartan both individually inducing a near-maximal effect on acetylcholine-mediated endothelium-dependent vasorelaxation. Nonetheless, a combination of exercise training and losartan may provide the better strategy for long-term HF treatment as it allows periods of absence of exercise without compromising endothelial function, as well as preserving the independent effects of each treatment alone beyond the endothelium (7-9).

Nitric oxide-dependent vasoregulation

By inhibiting eNOS-mediated NO-production by LNNA, prostacyclin-production by indomethacin, and sGC-cyclic guanosine monophosphate (cGMP)-dependent vasorelaxation by ODQ, the relative contributions of these pathways were assessed. This showed that impaired vasorelaxation in HF was due to reduced endothelial NO-availability, and that exercise training mainly improved vasorelaxation by stimulating NO-availability in both healthy and HF rats. This has also been suggested previously (1,6,13). Exercise training in combination with losartan also improved endothelial function via an NO-dependent pathway;

as shown previously (14,15), whereas in contrast, losartan alone did not stimulate NO-availability.

It has been suggested that eNOS-inhibition by L-arginine antagonists may only affect the stimulated and not the basal NO-production (16). In line with this, addition of SOD in the presence of LNNA; an irreversible inhibitor with a slow dissociation rate (17), still stimulated the vasorelaxation, except in losartan-treated sedentary HF rats. This points to an inability to completely block the basal NO-production, but also suggests that losartan upregulated an endothelial-mediated NO- and prostacyclin-independent vasodilator, for which the molecular identity remains unknown, but may be EDHF. As such, although the combination of exercise training and losartan did not provide observable additive effects, the combination strategy may be important inasmuch as previous experiments have suggested that the exercise-induced NO-dependent vasodilation varies greatly and exists only transiently after an exercise session (18). Losartan may thus provide an additional pathway of vasorelaxation when the NO-mediated regulation is low.

Endothelial-mediated NO- and prostacyclin-independent vasorelaxation

The foremost candidate for endothelial-mediated NO- and prostacyclin-independent vasoregulation is EDHF, but its molecular identity remains unclear and may encompass several factors originating from the endothelium (19); of which hydrogen sulfide (H₂S) is a recent candidate (20). In HF, the contribution of EDHF relative to NO for inducing vasorelaxation is increased, partly because NO is reduced (21). This was also indicated in our study. The finding that losartan exerted an endothelium-dependent, but NO- and prostacyclin-independent effect, i.e. an effect attributable to stimulated EDHF, is similar to that observed after angiotensin-converting enzyme (ACE) inhibition (22). Together, this suggests an

angiotensin II action that is not confined to the vascular smooth muscle cell, but may also involve the endothelium.

Since blockade of the sGC-cGMP pathway by ODQ effectively removed both NO-dependent and endothelium-dependent but NO- and prostacyclin-independent; thus, possibly EDHF-mediated, vasorelaxation, it opens up the possibility that NO and EDHF or its equivalent may initiate similar sGC-cGMP-dependent reactions in the smooth muscle cell. This is in line with reports of angiotensin II negatively affecting sGC independent of NO (23). Finally, we also observed that the NO- and prostacyclin-independent contribution to endothelium-mediated vasorelaxation was reduced when exercise training was introduced to HF rats on losartan, indicating that activated NO prevailed as the main vasodilator.

Conclusion

Endothelial dysfunction in HF was reversed by treatments involving exercise training and the ARB losartan, either alone or in combination. However, whereas exercise training improved endothelial function by restoring NO-production, losartan upregulated a different intra-endothelial pathway that is NO- and prostacyclin-independent. When losartan and exercise training were combined, no additional vasorelaxation occurred, but NO prevailed as the main vasodilator. Nonetheless, losartan might activate an additional mechanism for vasorelaxation that supplements NO-dependent vasorelaxation, which may be important when and if NO production is compromised.

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CONFLICT OF INTEREST

None.

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TABLES

Table 1. Experimental interventions.

A; Group assignment				
	Heart failure	Losartan	Exercise training	
SH SED PL	-	-	-	
SH EX PL	-	-	+	
HF SED PL	+	-	-	
HF SED LOS	+	+	-	
HF EX PL	+	-	+	
HF EX LOS	+	+	+	
B; Pharmacological interventions during recordings of vascular function				
	Pre-incubation	Contraction	Relaxation	2 nd Pre-incubation
A	3 mM L-Arg, 10 ⁻⁵ M Indo	3·10 ⁻⁷ M Phe	10 ⁻⁹ -3·10 ⁻⁵ M Ach	10 ⁻⁵ M ODQ
B	250 IU SOD, 10 ⁻⁵ M Indo	3·10 ⁻⁷ M Phe	10 ⁻⁹ -3·10 ⁻⁵ M Ach	10 ⁻⁴ M LNNA
C	250 IU SOD	3·10 ⁻⁷ M Phe	10 ⁻⁹ -3·10 ⁻⁵ M Ach	10 ⁻⁵ M ODQ
D	10 ⁻⁵ M Indo	3·10 ⁻⁷ M Phe	10 ⁻⁹ -3·10 ⁻⁵ M Ach	10 ⁻⁴ M LNNA
E	Vehicle	3·10 ⁻⁷ M Phe	10 ⁻⁹ -3·10 ⁻⁵ M Ach	10 ⁻⁴ M LNNA
F	Vehicle	3·10 ⁻⁷ M Phe	10 ⁻⁹ -3·10 ⁻⁵ M SNP	10 ⁻⁵ M ODQ

SH: sham operation; SED: sedentary; PL: placebo; EX: exercise training; HF: heart failure; LOS: losartan; L-Arg: L-arginine; Indo: indomethacin; SOD: superoxide dismutase; Phe: phenylephrine; Ach: acetylcholine; SNP: Na⁺ nitroprusside; ODQ: 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; LNNA: N^G-nitro-L-arginine.

Table 2. Left ventricular pressure recordings and infarct sizes.

	Sham		HF			
	SED-PL	EX-PL	SED-PL	SED-LOS	EX-PL	EX-LOS
LVEDP (mmHg)	4±1*	4±2*	26±2	19±1‡	22±1	17±1†
LVPSP (mmHg)	114±5*	120±6*	100±3	87±5	98±3	88±5
MI/LV (%)			43±3	46±5	36±3	40±3

Pressures were measured at the end of the intervention period, whereas infarct size was measured one week after surgery. LVEDP: left ventricular end-diastolic pressure; LVPSP: left ventricular peak-systolic pressure; MI/LV: size of myocardial infarction (MI relative to the left ventricle (LV). HF: heart failure; SED: sedentary; PL: placebo; EX: exercise training; LOS: losartan. Sham vs. HF: * $p<0.01$; HF-EX-LOS vs. HF-SED-PL: † $p<0.01$; HF-SED-LOS vs. HF-SED-PL: ‡ $p<0.05$.

FIGURE LEGENDS

Figure 1. Maximal oxygen uptake (VO_{2max}) as a measure of exercise capacity. VO_{2max} in sham-operated (SH) rats was 40% higher (* $p<0.01$) than in those with heart failure (HF) before the exercise training program (pre-test). Post-tests indicated that VO_{2max} increased 40% by exercise training (EX) in SH and HF rats; † ($p<0.01$), and by 20% in losartan (LOS)-treated sedentary (SED) HF rats compared to placebo (PL)-controlled HF rats; ≠ ($p<0.01$), respectively. Note that HF EX PL and HF EX LOS curves sit on top of each other. Also, SH SED PL and HF SED PL rats decreased VO_{2max} from pre-to post-tests; # ($p<0.05$).

Figure 2. A: Heart failure (HF) reduced the acetylcholine (Ach)-mediated vasorelaxation of aorta as indicated by the right shifted dose-response curve of sedentary (SED) HF rats on placebo (PL), whereas exercise training and losartan corrected for this dysfunction. B: Sensitivity to acetylcholine (EC_{50}); note the y-axis has a logarithmic scale. Aorta segments of the SED HF rats relaxed less than all the other groups (* $p<0.05$). Interventions with exercise training (EX), losartan (LOS) or a combination of both (EX LOS) improved endothelial sensitivity to Ach to levels comparable with SED sham-operated (SH) rats, whereas SH EX rats exceeded SED SH rats († $p<0.01$). C: Maximal vasorelaxation was increased in LOS-treated SED HF rats compared to PL-controlled SED HF rats (* $p<0.05$). D: EC_{50} -values of Ach-mediated vasorelaxation (white and black bars), with delta EC_{50} -values (grey bars) indicating difference in Ach-mediated vasorelaxation after addition of 3 mM L-arginine; this maneuver increased the response in HF-SED-PL (* $p<0.05$). E: EC_{50} -values of Ach-mediated vasorelaxation (white and black bars), with delta EC_{50} -values (grey bars)

indicating difference in Ach-mediated vasorelaxation after addition of 250 IU superoxide dismutase (SOD); this maneuver increased the responses in SH-SED-PL and HF-SED-PL (* $p<0.05$ and † $p<0.01$).

Figure 3. A: Acetylcholine (Ach)-mediated vasorelaxation in the presence of 10^{-5} M indomethacin (Indo). B: Ach-mediated vasorelaxation in the presence of 10^{-4} M LNNA and 10^{-5} M Indo, with a trend ($p=0.12$) towards an increase in vasorelaxation in the heart failure (HF) rats on losartan (LOS) vs. placebo (PL)-controlled sedentary (SED) or exercise training (EX) HF or sham-operated (SH) rats. C: Ach-mediated vasorelaxation in the presence of 10^{-5} M ODQ and 10^{-5} M Indo. D: Vasorelaxation due to nitric oxide (NO) was calculated by subtracting the area under curve (AUC) of the graphs in panels B and C from the AUC of panel A ($A-(B+C)$). HF-EX-LOS vs. HF-SED-PL and HF-SED-LOS: * $p<0.05$; SH-EX-PL and HF-EX-PL vs. HF-SED-LOS, HF-SED-PL and SH-SED-PL: † $p<0.01$.